

Aus dem Institut für Humangenetik und Anthropologie
der Universität Erlangen-Nürnberg
Direktor: Professor Dr. med. G. Koch

**Die Beeinflussung der chromosomenschädigenden
Aktivität von Trenimon^R in menschlichen
Lymphozyten in vitro durch Reducdyn^R**

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Margarita Maria Stosiek
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Protective Effect of Reducdyn® against the Chromosome Damaging Activity of 2,3,5-Triethyleimine-Benzoquinone-1,4 on Human Lymphocytes *in vitro**

Margitta Stosiek* and Erich Gebhart

Institut für Humangenetik und Anthropologie der Universität Erlangen-Nürnberg
(Direktor: Prof. Dr. G. Koch)

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Summary. The protective effect of the commercial radioprotective drug Reducdyn® (components: N-acetyl-homocysteine-thiolactone, cysteine, and fructose) was tested against the chromosome damaging activity of 2,3,5-triethyleimine-benzoquinone-1,4 (Trenimon®) in human lymphocytes *in vitro*. Simultaneous application of Reducdyn® distinctly reduced the number of Trenimon-induced chromosome aberrations. The protective efficacy was dependent on concentration. It varied according to different types of aberrations. The cytostatic action of Trenimon (measured by estimation of the mitotic indices) was not affected by the protective drug. Pretreatment with Reducdyn® also yielded a protective activity, although to a lesser degree than the simultaneous application with clastogen. Post-treatment with the protective drug was ineffective. Both the theoretical and practical aspects of the results are discussed.

1. Introduction

Protectors against the toxic but also against the mutagenic or chromosome damaging activity of ionizing radiations have been known for many years and have been tested in various organisms and test systems (Bacq, 1965; Rieger and Michaelis, 1967). Several investigations on the protective activity of radioprotectors on the mutagenic or clastogenic action of chemicals yielded good results in plant cells, *Drosophila*, and also human lymphocytes *in vitro* (see Gebhart, 1974 for references). In human lymphocytes the SH-containing amino acid, cysteine, clearly reduces the number of chromosome aberrations induced by busulphan (Gebhart, 1969), Trenimon® (Gebhart, 1970a, 1971a), 8-oxyquinoline sulfate (Gebhart, 1971b), Thiotepe (Arutunian and Kuleshov, 1972), and cytosine arabinoside (Arutunian, 1973). The biogenic amine cysteamine (mercaptoethylamine) and the radioprotector β -aminoethyl-isothiourea (AET) also display a protective effect against the chromosome damaging activity of Trenimon® (Becher and Gebhart, 1973). Therefore, the question as to whether mixing of protectors yields a better protective effect needed clarifying urgently.

The radioprotective drug Reducdyn® has been used for several years in the therapy of radiation disease as well as various liver diseases. It is composed of N-acetyl-D,L-homocysteine-thiolactone (AHT), L-cysteine, and D-fructose.

Tests on the anticlastogenic activity of this mixture against a chemical clastogen seemed necessary not only for fundamental but also for practical reasons. Trenimon® (2,3,5-triethyleneimine-benzoquinone-1,4) seemed to be the most suitable clastogen because of its use as a cytostatic drug as well as its well-analyzed clastogenic activity (Hampel, 1968; Gebhart, 1970a). Studies on the influence of Reducdyn on the chromosome damaging activity of Trenimon in human lymphocyte cultures *in vitro* should not only clarify a potential anti-clastogenic effect after simultaneous application of the presumable protector and the clastogen. Suitable tests could also show whether pre-treatment or post-treatment influences Trenimon-induced aberration rates.

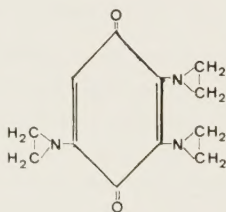
2. Material and Methods

All tests were performed in 3-day cultures of human peripheral blood lymphocytes according to a modified method of Moorhead *et al.* (1960) presented in earlier publications (e.g. Gebhart, 1970a; Becher and Gebhart, 1973). In contrast to previous studies Ham's F 10 (Biocult) was used as a culture medium.

The test substances are shown in Fig. 1. The mixing ratio of the components of Reducdyn (for injections) is 1 (AHT): 3 (cysteine): 40 (fructose). The test substances were dissolved and diluted in bi-distilled water and added to the cultures in quantities not exceeding 0.2 ml.

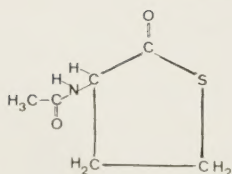
1. *Simultaneous Application of Reducdyn and Trenimon for 24 Hrs.* The final concentration of Trenimon in these tests was 10^{-7} mol/l, while Reducdyn was tested in three final concentrations: a) 10^{-4} mol/l corresponding to AHT, b) 10^{-4} mol/l corresponding to cysteine, and c) 10^{-4} mol/l corresponding to fructose.

2. *Pretreatment with Reducdyn for 18 Hrs Followed Up by Application of Trenimon for 24 Hrs.* The final concentrations in these tests were 10^{-4} mol/l corresponding to AHT for Reducdyn and 10^{-7} mol/l for Trenimon again.

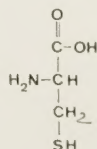


TRENIMON[®]

COMPONENTS OF REDUCDYN[®]



N-ACETYL-L-HOMOCYSTEINE-THIOLACTONE



L-CYSTEINE

Fig. 1. Test substances (a further component of Reducdyn[®] is d-fructose)

3. *Post-treatment with Reducdyn for 18 Hrs of Cells Pre-treated with Trenimon for 24 Hrs.* The final concentrations in these tests were the same as in the pre-treatment tests.

4. *20 Hrs Recovery between 24 Hrs Pre-treatment with Trenimon and Post-treatment with Reducdyn* (final concentrations as in 2). For every tests stage a minimum of 200 well-spread metaphases from 4 different cultures of 2 independent test series were analyzed for structural chromosome aberrations, according to previously defined criteria (Gebhart, 1970b).

The protective efficacy was depicted as "reduction factor" (RF) which is the quotient of the number of observed aberrations after a combined treatment with protector and clastogen and the number of observed aberrations after treatment with the clastogen only.

3. Results

3.1. Cytogenetic Activity of Trenimon® and Reducdyn®

The chromosome damaging activity of Trenimon has already been analyzed extensively (Hampel, 1968; Gebhart, 1970a; Becher and Gebhart, 1973). The qualitative data of this study are in keeping with the former results. The quantitative analysis, however, on average yielded higher aberration rates than were observed in previous papers. These deviations may easily be explained by the use of another culture medium (Ham's F 10, former tests: Difo TC 199).

Chromosome analyses in cultures treated with Reducdyn only revealed no clastogenic activity of the drug: while the number of chromatid and isochromatid breaks was in the range of untreated control cultures, the number of chromatid and isochromatid gaps was slightly increased.

3.2. Simultaneous Application of Reducdyn® and Trenimon®

Table 1 represents the main data obtained after treatment of the lymphocyte cultures simultaneously with Reducdyn and Trenimon for 24 hrs. A clear protective effect of Reducdyn is shown which was characterized by a mean RF value of 0.54 at the highest concentration (10^{-4} mol/l corresponding to AHT). At a concentration of 10^{-4} mol/l corresponding to cysteine the mean reduction factor was 0.77 and at the lowest concentration (10^{-4} mol/l corresponding to fructose) it still was 0.84. Fig. 2 shows the RF values of the single types of aberrations. A clear dependence of the protective efficacy from the Reducdyn concentration is obvious with chromatid gaps as well as with chromatid and isochromatid breaks and chromatid interchanges.

Table 1. Influence of simultaneous application of Reducdyn® at various concentrations on the cytogenetic action of Trenimon® (10^{-7} m)

Trenimon Reducdyn	—	+	—	+	—	+	—	+
	—	—	+1	+1	+2	+2	+3	+3
Analyzed metaphases	200	200	200	200	200	200	200	200
Metaphases with aberrations (%)	10	90.5	18	78	16	82.5	13.5	81
Metaphases with breaks (%)	6.5	79.5	7	63.5	7.5	70	6.5	76
Gaps per metaphase	0.04	0.81	0.15	0.53	0.11	0.58	0.08	0.58
Breaks per metaphase	0.07	2.1	0.07	1.17	0.08	1.82	0.05	2.05

— = without; + = with; +1 = with 10^{-4} mol/l (corresp. to AHT),
 +2 = with $2.5 \cdot 10^{-5}$ mol/l (corresp. to AHT),
 +3 = with $3.3 \cdot 10^{-6}$ mol/l (corresp. to AHT).

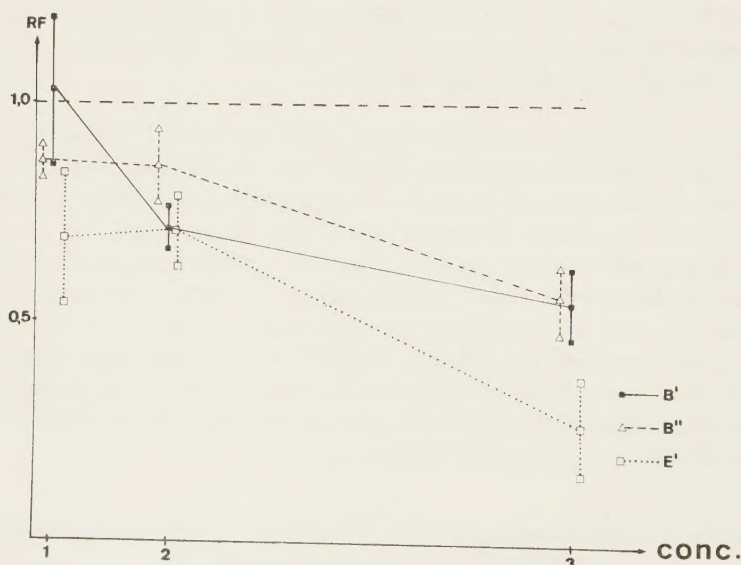
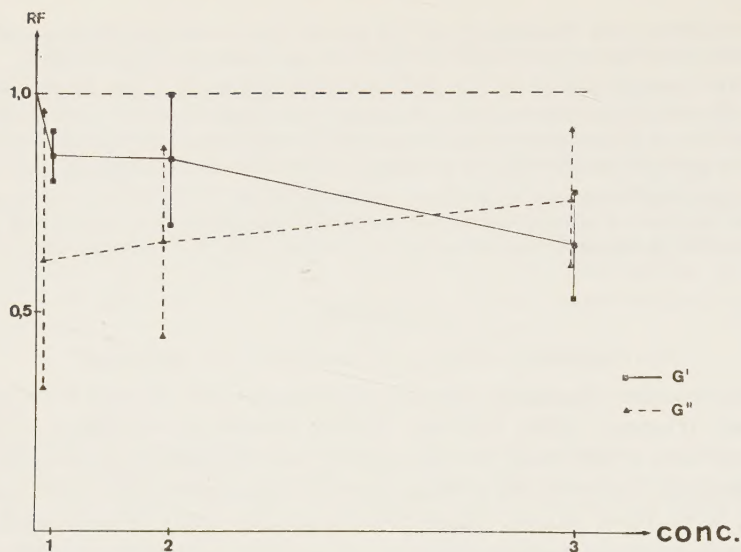
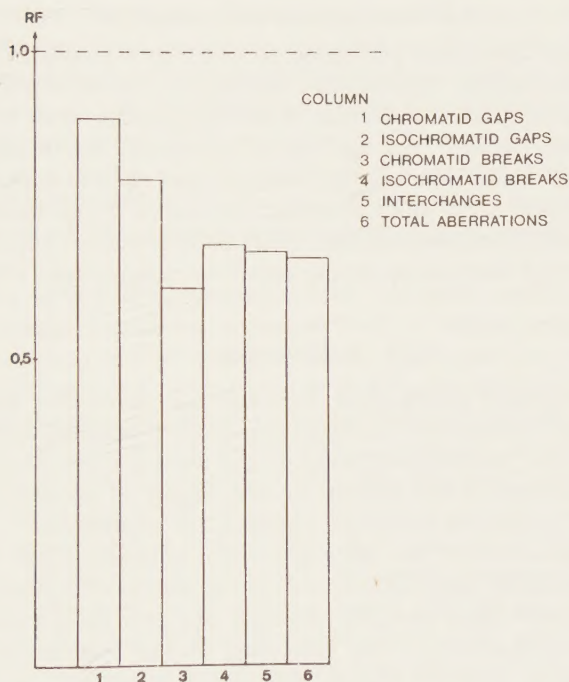


Fig. 2. Dependence from the concentration of the protector effect of Reducdyn® simultaneously applied with Trenimon® (10^{-7} mol/l) for 24 hrs. Conc. 1: $3.3 \cdot 10^{-6}$ mol/l; conc. 2: $2.5 \cdot 10^{-5}$ mol/l; conc. 3: 10^{-4} mol/l (all corresp. to AHT). G' chromatid gaps; G'' isochromatid gaps; B' chromatid breaks; B'' isochromatid breaks; E' chromatid interchanges

For the examination of a possible influence of Reducdyn on the mitotic activity of Trenimon-treated lymphocytes, the mitotic indices were estimated in all cultures treated with Reducdyn and Trenimon as well as in cultures treated with Reducdyn or Trenimon only, and in untreated control cultures. No deviation of mitotic indices was observed under the influence of Reducdyn.

Table 2. Influence of 18 hrs pretreatment with Reducdyn® (10^{-4} mol/l corresp. to AHT) on the cytogenetic action of Trenimon® (24 hrs 10^{-7} mol/l)

Trenimon	—	+	—	+
Reducdyn	—	—	+	+
Analyzed metaphases	200	200	200	200
Metaphases with aberrations (%)	12	92	11	77
Metaphases with breaks	7	82	7	64.5
Gaps per metaphase	0.07	0.63	0.10	0.46
Breaks per metaphase	0.08	2.27	0.08	1.52

Fig. 3. Effect of 18 hrs pre-treatment with Reducdyn® (10^{-4} mol/l corresp. to AHT) on the Trenimon-induced aberration rate

3.3. Pre-treatment with Reducdyn®

Another series of tests was performed to ascertain whether chromosome structure is protected by simultaneous application of Reducdyn and Trenimon only, or whether a prophylactic protection is also possible. As shown in Table 2, pre-treatment with Reducdyn also distinctly reduces the aberration rates induced by Trenimon. The mean RF value in these tests is about 0.7. As after simultaneous treatment, pre-treatment with Reducdyn yields different protective activity depending on the respective type of aberration (Fig. 3).

Table 3. Influence of 18 hrs post-treatment with Reducdyn® (10^{-4} mol/l corresp. to AHT) on the cytogenetic action of Trenimon® (24 hrs; 10^{-7} mol/l)

Trenimon	—	+	—	+
Reducdyn	—	—	+	+
Analyzed metaphases	300	300	300	300
Metaphases with aberrations (%)	6	31	7	26
Metaphases with breaks (%)	1	24	2	20
Gaps per metaphase	0.05	0.11	0.05	0.09
Breaks per metaphase	0.01	0.32	0.02	0.31

3.4. Post-treatment with Reducdyn®

Post-treatment tests were performed to clear up a possible reparative effect of Reducdyn on manifest chromosome aberrations. As shown in Table 3, these tests revealed no significant reduction of the cytogenetic effect of the alkylating agent. It was conspicuous that the rate of chromatid interchanges in cultures post-treated with Reducdyn slightly exceeds that in cultures with Trenimon only. In tests, where there was a recovery interval of 20 hrs between the 24 hrs pre-treatment with Trenimon and the 18 hrs post-treatment with Reducdyn, the number of observed aberrations was too small for a significant estimation of RF values.

4. Discussion

The data presented above show that the radioprotective drug Reducdyn® distinctly reduces chromosome damage induced by Trenimon® in human lymphocytes *in vitro*. The protective efficacy of the anticlastogen clearly depends on its final concentration in the culture, on the timing of application, and on the respective type of induced structural chromosome aberration.

The dose dependence of the protective effect is nearly linear with respect to breaks of the chromatid and isochromatid type as well as with respect to chromatid gaps. The protective effect certainly does not originate from a direct reaction of the components of Reducdyn with Trenimon. As has been demonstrated in this study as well as in previous studies with other protectors (see Gebhart, 1974 for references) a considerable excess of the protective drug over the concentration of the clastogen is necessary to display a distinct protective activity. If the protector reacted with the clastogen directly, a total inactivation of the whole amount of the clastogen would be expected, resulting in a decrease of the aberration rates to the range of controls. The protective effect observed in this study and in previous tests, however, was in no case a total one but was always relative. The fact that the antimutagenic activity of Trenimon is not influenced by Reducdyn provides further evidence that there is no direct reaction between the components of Reducdyn® and Trenimon®.

Simultaneous application of the protective drug with the clastogen yielded the clearest anticlastogenic effect, but pre-treatment with Reducdyn also reduced Trenimon-induced aberration rates to a remarkable extent. In contrast, post-treatment with Reducdyn of cells previously damaged by Trenimon seemed to

be ineffective. In earlier studies with cysteine (Gebhart, 1971a) pre- and post-treatment tests yielded no significant results. The absence of a pre-treatment effect in this case may be due to the low stability of the SH group of cysteine in such a long-term experiment. The data obtained with Reducdyn suggest that its components might be concentrated in the cells in a more stable form. The fact that post-treatment fails to show a protective efficacy demonstrates that subsequent repair of aberrations just induced by the anticlastogens is not possible.

The observation that different types of aberration are influenced at different degrees may contribute to a better understanding of the anticlastogenic action of Reducdyn or its components. Under the influence of Reducdyn the number of Trenimon-induced breaks is reduced to a greater extent than is that of gaps; chromatid aberrations are more distinctly suppressed than isochromatid aberrations. In contrast to other protectors examined earlier, Reducdyn reduced the number of Trenimon-induced chromatid interchanges to below one third of the number in cells treated only with Trenimon. These data do not favor a stabilizing or even a strengthening effect of the components of Reducdyn on enzyme systems engaged in reunion (repair?) of open breaks, as it was demanded on the basis of data obtained with cysteamine (Becher and Gebhart, 1973) or amino acids (Gebhart, 1973). A more reliable explanation in the case of Reducdyn is a real reduction of breakage.

The fact that in the tests presented here the total number of Trenimon-induced chromosome aberrations is higher than in earlier comparable tests is explained by the use of another culture medium. OBE (personal communication) has shown that different cell populations with different sensitivity to clastogens may be obtained at the same culture time after culturing in different media.

No conclusive results may be drawn from cytogenetic experiments alone as to the mechanisms of protective action of Reducdyn. Nevertheless our data suggest that the stabilization of the SH group by forming a lactone ring like in AHT may display an important role in the extent of the protective effect. In addition to the relatively short-lived SH groups of cysteine a subsequent production of SH groups by hydrolysis of the thiolactone ring of AHT (Braun *et al.*, 1957) may not only strengthen but also prolong the protective activity of cysteine.

There is a large amount of literature on the possible role of SH groups in radio- and chemoprotection (see Gebhart, 1974 for references), so that on the basis of our data our considerations on this topic should be limited to the above statements. Further tests on the protective effect of AHT which just started may present further data concerning the possible mechanisms of protection.

After all, we are sure that the observations on the protective action of Reducdyn support the assumption that drugs containing one or several SH compounds will be a source of antimutagenic and anticlastogenic agents respectively for practical use. In this context Reducdyn may be an essentially favorable drug because it has been in practical use for many years and has proven its good compatibility with man. On the other hand the necessity for radio- and chemotherapeutic treatment of a steadily increasing group of persons with mutagenic and clastogenic agents respectively urges us to find practically applicable protectors. Of course a series of further tests especially in mammalian *in vivo* test systems will be necessary before definite statements on antimutagenic and anticlastogenic

action in man will be possible. The first results of anticlastogen research seem to be encouraging enough to pursue and to intensify the search for antimutagens of practical applicability in man. A study on the anticlastogenic effect of Reducdyn in patients undergoing cytostatic therapy just has been started.

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Margitta Stosiek
 PD Dr. E. Gebhart
 Institut für Humangenetik und Anthropologie
 der Universität
 D-8520 Erlangen, Bismarckstraße 10
 Federal Republic of Germany

Lebenslauf

Am 17. Juli 1948 wurde ich, Margarita Maria Stosiek, als zweites von sechs Kindern des Arztes für Allgemeinmedizin Dr. Karl Stosiek und seiner Ehefrau Felicitas Stosiek, geb. Tenschert, in Langenthal (Opf.) geboren. Von 1954 bis 1958 besuchte ich die Volksschule in Berg im Landkreis Neumarkt/Opf. Im September 1958 wechselte ich auf das Willibald-Gluck-Gymnasium Neumarkt/Opf. über, wo ich 1967 die Reifeprüfung ablegte.

Vom Wintersemester 1967/68 bis Wintersemester 1971/72 studierte ich an der Universität Erlangen-Nürnberg Chemie und Biologie. Im Dezember 1971 bestand ich die Wissenschaftliche Prüfung für das Lehramt an Gymnasien in Chemie und Biologie.

Im Sommersemester 1971 begann ich mit dem Studium der Medizin an der Universität Erlangen-Nürnberg. Im Juni 1972 legte ich die naturwissenschaftliche und im Oktober 1972 die ärztliche Vorprüfung ab. Am 28. Januar 1976 bestand ich das Medizinische Staatsexamen.

Erlangen, im Februar 1976

Margarita Maria Stosiek

